INVESTIGATION OF THE FERTILIZATION INHIBITING ACTION OF ARBACIA DERMAL SECRETION [1]

CHARLES B. METZ

Oceanographic Institute, Florida State University, Tallahassee, Florida and Marine Biological Laboratory, Woods Hole, Mass.

Several investigations (Oshima, 1921; Pequegnat, 1948; Harvey, 1956; Metz, 1959a) have shown that *Arbacia* releases a fertilization inhibiting agent or agents when subjected to conditions of stress. The agent is present in a yellowish fluid called "dermal secretion" (Oshima, 1921) and is probably released from certain pigment-containing cells of the integument (Pequegnat, 1948). Indeed this dermal secretion is probably responsible for the fertilization inhibiting action ascribed to *Arbacia* blood by Lillie (1914).

Unfortunately, little information is as yet available concerning the mechanism by which the dermal secretion inhibits fertilization. In a previous study (Metz, 1959a) it was found that the dermal secretion inhibits the agglutination of *Arbacia* sperm by fertilizin, the specific sperm isoagglutinin obtained from the jelly layer surrounding eggs. This agglutination inhibiting action was found to result from an inactivation of the specific combining sites of the fertilizin that react with the antifertilizin of the sperm surface.

The parallel inhibiting action of dermal secretion on fertilizin agglutination of sperm and on fertilization suggests that the latter action may also result from inactivation of fertilizin combining sites. The present study was undertaken to investigate the mechanism of fertilization inhibition by the dermal secretion and particularly to determine if this action resulted from an inactivation of fertilizin. The experiments have been reported briefly elsewhere (Metz, 1959b). They indicate that fertilizin is not the site of inhibitor action.

Materials and Methods

Two species of sea urchins were used in the experiments, namely *Lytechinus variegatus* and *Arbacia punctulata.* Animals of the former species were obtained from the large population in the vicinity of the Florida State University Marine Laboratory, Alligator Point, Florida. The latter (*Arbacia*) were also obtained from this area, and from Panama City, Florida. This species was also obtained from the Woods Hole, Massachusetts, area and used at the Marine Biological Laboratory.

Gametes were obtained from *Lytechinus* by inducing the animals to shed by treatment with 0.5 *M* KCl. Gametes were obtained from *Arbacia* by electrical stimulation.

[1] Aided by grants from the National Institutes of Health and the National Science Foundation. Contribution #108 from the Oceanographic Institute, Florida State University.

Solutions of dermal secretion were obtained by immersing *Arbacia* in tap or distilled water for 1–3 minutes, rinsing them in sea water and collecting the yellowish fluid that drained from the animals (Metz, 1959a). The pH of such dermal secretion is about 7.5. The pH was adjusted to that of sea water (8.0–8.2) before use.

Results

In the presence of *Arbacia* dermal secretion both *Arbacia* and *Lytechinus* eggs fail to fertilize, even though these eggs are bombarded by highly motile spermatozoa. Such eggs fail to elevate fertilization membranes or show any indication of nuclear activity. Clearly, then, the dermal secretion blocks fertilization at its initial stages. The first step in analyzing the mechanism of this action of the

Table I

Percentage of eggs cleaving after insemination with Arbacia *sperm washed from dermal secretion*

Dilution of treated sperm	Dermal secretion—treated sperm		S.W.—treated sperm	
	Washed in S.W.	Re-suspended in D.S. supernatant	Washed in S.W.	Re-suspended in S.W. supernatant
1	100% (200+)	60% (103)	100% (200+)	100% (200+)
1/5	100% (200+)	100% (200+)	100% (200+)	100% (200+)
1/25	90% (128)	100% (200+)	99% (200+)	100% (200+)
1/125	62% (98)	100% (97)	97% (119)	100% (200+)
1/625	20% (109)	61% (116)	64% (108)	93% (123)

Figures in parenthesis are the number of eggs counted. The four mixtures were prepared in the following proportions: 0.5 ml. approximately 20% sperm suspension; 1.5 ml. D.S. or S.W. These mixtures were centrifuged and the sperm suspended in 1.5 ml. S.W. or in the supernatant. This procedure was repeated and all four sperm suspensions were finally diluted with 25 ml. S.W. (sperm "dilution 1" = approximately 0.4% undiluted semen). The suspensions were diluted serially as indicated. Two drops of unfertilized eggs were added to 4 ml. of each sperm dilution. In a control experiment eggs failed to fertilize when inseminated in the D.S. supernatant from the centrifuged sperm suspension.

dermal secretion was to determine if the agent acted upon the sperm, the egg, or both gametes.

Action on the sperm. In the previous studies (Pequegnat, 1948; Metz, 1959a) it was found that the dermal secretion from *Arbacia* markedly enhanced both motility and respiration of *Arbacia* sperm and that these effects were long lasting. Evidently, then, any fertilization inhibiting action on the sperm must result from some subtile effect such as "premature discharge" of the acrosomal filament or blocking of an essential receptor site on the sperm surface.

To test for such action *Arbacia* sperm were treated with dermal secretion, subsequently washed free of the solution by centrifugation and finally tested for fertilizing capacity. As seen in Table I, *Arbacia* sperm washed from dermal secretion are as effective in fertilizing eggs as sperm washed from sea water. Furthermore, dilution of dermal secretion-treated sperm with sea water restores fertilizing capacity. Thus (Table I) the sperm in dermal secretion gave only 60% fertilization at "dilution 1" but yielded 100% fertilization at higher dilutions.

This inhibition at "dilution 1" is attributed to an action of dermal secretion on the eggs. At the higher dilutions the dermal secretion is evidently diluted beyond the minimum inhibitory concentration. Finally, the data suggest that washing, as opposed to re-suspension in the supernatant, reduces the fertilizing capacity of control sperm. This is in accord with Hayashi's (1945) observations on the beneficial effect of seminal plasma on sperm. Results similar to those in Table I were obtained in a second experiment using *Arbacia* sperm and eggs. Comparable results were also obtained in two experiments with *Lytechinus* sperm and eggs using *Arbacia* dermal secretion. However, the results were not as striking in these experiments because the more delicate *Lytechinus* sperm does not survive centrifugation and re-suspension as well as *Arbacia* sperm.

It is evident from these experiments that dermal secretion does not inhibit fertilization by irreversible action on the sperm. Accordingly, attention was directed toward possible effects on the egg.

Action of dermal secretion on the egg. According to Pequegnat (1948) washing in sea water at least partially restores fertilizability to dermal secretion-treated *Arbacia* eggs. Harvey (1956, page 57), on the other hand, states that "it has been my experience that sometimes the eggs are not fertilizable, even after repeated washings with sea water." Observations in the present study indicated that reversibility of inhibition by washing varies from preparation to preparation. Eggs washed from some dermal secretion preparations fertilize readily whereas those from others show varying degrees of reduced fertilizability. This is evidenced in two ways. As seen in Table III, experiment 1, eggs washed from a dermal secretion preparation may approach 100 per cent fertilizability. However, substantially higher sperm concentrations are required to fertilize such eggs than are required for the controls. A second expression of reduced fertilizability is delayed cleavage. In some experiments *Arbacia* eggs washed from dermal secretion and subsequently inseminated fail to cleave synchronously with the controls. In these, first cleavages are asynchronous and delayed one or two divisions as compared to the controls.

These results indicate that the probability of a "successful sperm-egg contact" (Rothschild, 1956) is lower for the treated eggs than for the control eggs under comparable conditions. The reduced probability of a successful sperm-egg contact in turn could result from a correspondingly reduced number of receptor sites on the egg surface. In the case of cleavage delay the treated eggs may not fertilize at the moment of insemination because time is required for a successful sperm-egg contact to be made. The assumption is then that the egg develops at the normal rate and cleaves on schedule following a successful sperm-egg contact. This view is supported at least to the extent that fertilized eggs show no cleavage delay when placed in the dermal secretion as early as one to two minutes following insemination. However, eggs placed in dermal secretion a few seconds after insemination frequently show arrested fertilization membrane elevation and sperm entry (especially clear in *Lytechinus* eggs). These observations suggest an inhibition of the cortical reaction. In any event these experiments show that washing does not readily restore dermal secretion-treated eggs to the control level of fertilizability. Accordingly, it appears likely that the dermal secretion inhibits fertilization by an effect upon fertilizability of the eggs, as opposed to sperm. This view is supported by studies with enzymes as recorded in a later section.

The effect of dermal secretion on the formation of blebs by inseminated oocytes. The observations described above suggest that the dermal secretion inhibits, blocks or otherwise prevents an early step in fertilization. In a further test of this assumption dermal secretion was examined for its effect on the formation of blebs or papillae on oocytes when these cells are inseminated. As is well known (*e.g.*, Rothschild and Swann, 1949) sea urchin oocytes are not fertilizable. However, these cells, when exposed to sperm, form numerous blebs on their surfaces. Formation of such blebs has been attributed to an interaction between the sperm and oocyte surface. To test for the effect of dermal secretion on oocyte bleb formation, suspensions of eggs containing oocytes were placed in dermal secretion solutions, sperm was then added and the mixtures examined for bleb formation and compared with appropriate controls. Many such observations using both *Arbacia* and *Lytechinus* eggs show that in the presence of dermal secretion bleb formation does not result to a significant degree, whereas numerous blebs formed in the control oocytes. Some inhibitory reaction evidently results which prevents interaction of sperm and oocyte or blocks the bleb-forming response mechanism of the oocyte. In any event these observations support the view that the dermal secretion blocks an early step in fertilization.

In a further attempt to localize the site of action of the dermal secretion, the egg surface was subjected to mild dissection and then tested for sensitivity to the inhibiting action of the dermal secretion.

The effect of dermal secretion on fertilizability of acid-treated eggs. The first agent selected for such dissection was acid sea water. As is well known (Tyler, 1948) acid sea water dissolves the jelly surrounding the egg and thereby removes the fertilizin from the egg except possibly for a layer bound to the egg surface. In two experiments *Lytechinus* eggs were exposed to pH 4–5 sea water and in four experiments *Arbacia* eggs were exposed to pH 5 sea water to remove the jelly layer. These eggs were subsequently washed in sea water, treated with dermal secretion and in some cases washed again and tested for fertilizability over a wide range of sperm concentrations. In all experiments the acid-treated eggs showed the same sensitivity to the fertilization inhibiting action of dermal secretion as did control eggs. Table II gives two experiments, one using *Arbacia* eggs and the other *Lytechinus* eggs. In both experiments it is clear that acid-treated and sea water-treated eggs are both sensitive to the fertilization inhibiting action of the dermal secretion. The well known reduction in fertilizing capacity upon removal of jellies from eggs (Tyler, 1948) is not pronounced at the sperm concentrations employed but indications of this effect are seen in the experiment with *Lytechinus* eggs.

Since the sensitivity of eggs to the fertilization inhibiting action of the dermal secretion is not affected by acid treatment, it follows that the site of action of the dermal secretion is not the egg jelly. It is also presumably not the egg fertilizin since fertilizin constitutes the jelly. However, it is possible that a layer of fertilizin remains bound at the egg surface after the acid treatment and that this is an essential layer blocked by the dermal secretion (*e.g.* Tyler, 1948).

The effect of dermal secretion on fertilizability of eggs treated with proteolytic enzymes. Proteolytic enzymes were used to achieve a further mild dissection of the egg surface. As is well known (*e.g.* Tyler and Metz, 1955; Tyler, Monroy and Metz, 1956), eggs treated with proteolytic enzymes lack jellies, they fail to

form normal fertilization membranes, they become polyspermic readily and hybridize more readily than control eggs when inseminated with sperm of foreign species. The following proteolytic enzyme preparations were used: "crystalline protease" (Nutritional Biochemical Corporation) in 0.1% sea water solution; "crystalline trypsin" (Nutritional Biochemical Corporation) in 0.01% sea water solution. Enzymatic action of the trypsin was stopped prior to addition of dermal secretion by washing the eggs in an excess of trypsin inhibitor (ovomucoid, Nutritional Biochemical Corporation, 0.1% solution in sea water; or soybean trypsin inhibitor, Mann Research Corporation, 0.1% in sea water). In all, ten experiments were performed. These included five with *Arbacia* eggs, one using protease and four employing the combination of trypsin and trypsin inhibitor. Experiments

TABLE II

Effect of Arbacia *dermal secretion on fertilizability of acid-treated eggs as indicated by per cent cleavage*

	Sperm dilution	Acid-treated eggs		S.W.-treated eggs	
		D.S.-treated	S.W.-treated	D.S.-treated	S.W.-treated
Arbacia eggs	1	23% (124)	91% (141)	15% (114)	90% (200+)
	1/5	5% (158)	97% (107)	2% (148)	90% (200+)
	1/25	1% (200+)	90% (152)	1% (200+)	90% (200+)
	1/125	0% (200+)	81% (128)	0% (200+)	97% (105)
Lytechinus eegs	1	14.5% (124)	88% (148)	19% (114)	96% (119)
	1/6	6% (114)	97% (147)	4.5% (150)	98% (134)
	1/36	0% (200+)	46% (140)	1% (200+)	35% (130)
	1/216	1% (200+)	14% (180)	0% (200+)	74% (182)

Arbacia eggs: Jellies were removed by acidification to pH 5.4, eggs were then washed twice in 50-ml. samples of S.W., 0.5 ml. eggs was added to 2.4 ml. D.S. or S.W. and finally three-drop samples of these eggs were added to 5-ml. samples of *Arbacia* sperm. Sperm dilution 1 = 0.1%.

Lytechinus eggs: Jellies were removed by acidification of pH 3.9. Eggs were washed twice in S.W., 0.75 ml. eggs was then added to 2 ml. D.S. or S.W. They were subsequently washed twice in S.W. Four drops eggs were added to 5 ml. of *Lytechinus* sperm. Sperm dilution 1 = 0.66%. D.S.-treated eggs at sperm dilution 1 showed high incidence of polyspermy. Many of the uncleaved eggs contained multipolar spindles.

using *Lytechinus* eggs included four applying protease treatment and one using the trypsin-trypsin inhibitor treatment. In all ten of these experiments enzyme-treated eggs were subsequently treated with dermal secretion (or sea water), washed in sea water and inseminated. With this procedure the enzyme-treated eggs fertilized equally readily whether they received the dermal secretion or sea water treatment. Proteolytic enzyme treatment alone, like acid treatment, lowers the fertilizing capacity of the eggs (*e.g.* Tyler and Metz, 1955). In Table III the results of two of the ten experiments, one using *Arbacia* and the other *Lytechinus* eggs, are presented. In both experiments the fertilizability of sea water-treated eggs was markedly reduced by treatment with dermal secretion. Thus in experiment 1 to even approach comparable fertilization (100% of the eggs), over 125 times the control sperm concentration was required to fertilize the dermal secretion-

treated eggs. In experiment 2 the difference in sperm concentration (to achieve 1% fertilization) was greater than 1000-fold. On the other hand no significant difference is seen between the fertilizability of dermal secretion and sea water-treated eggs that had been pretreated with enzyme. Indeed, comparison of the enzyme-treated and sea water-treated eggs shows that on an absolute basis enzyme

TABLE III

Effect of proteolytic enzymes on sensitivity of eggs to the fertilization inhibiting action of Arbacia *dermal secretion*

	Sperm dilution	Enzyme-treated eggs		S.W.-treated eggs	
		D.S.-treated	S.W.-treated	D.S.-treated	S.W.-treated
Experiment 1 trypsin-trypsin inhibitor-treated *Arbacia* eggs	1	99% (149)	97% (154)	89% (158)	100% (200+)
	1/5	98% (151)	68% (187)	91%‡ (162)	100% (200+)
	1/25	42% (295)	21% (186)	3% (156)	100% (200+)
	1/125	8% (199)	5% (187)	<1% (200+)	100% (200+)
Experiment 2 Protease-treated *Lytechinus eggs*	1	94% (107) (82%)*	93% (97) (51%)*	<1% (200+)	93% (88) (6%)*
	1/6	87% (118) (52%)*	83% (87) (47%)*	0% (200+)	98% (96) (2%)*
	1/36	81% (131) (13%)*	12% (149) (0%)*	0% (200+)	65% (113) (<1%)*
	1/216	37% (169) (<1%)*	25% (128) (<1%)*	0% (200+)	35% (108) (0%)*
	1/1296	14% (133)	1% (200+)	0% (200+)	<3% (200+)

% = Per cent cleavage.

Experiment 1. Two-ml. samples of *Arbacia* eggs were added to 4-ml. samples of S.W. and 0.05% trypsin (in S.W.). After 45 minutes the eggs were washed twice in S.W., 1.5 ml. of the eggs were transferred to 10 ml. 0.1% ovomucoid in S.W. Subsequently the eggs were washed again, samples were treated with D.S. and S.W. and washed. Four drops eggs were added to 5 ml. *Arbacia* sperm dilution series. A duplicate control experiment omitting the ovomucoid treatment gave similar results. Sperm dilution 1 = 0.1% semen. Degree of polyspermy was not recorded in this experiment. Figures in parenthesis are the total number of eggs counted.

‡ Cleavage was delayed two divisions as compared to controls.

Experiment 2. Five-tenths-ml. samples of unfertilized *Lytechinus* eggs were placed in 5 ml. 0.1% protease solution or S.W. After 89 minutes 1-ml. samples of protease- and S.W.-treated eggs were each added to 1-ml. samples of D.S. and S.W. Twenty minutes later the eggs were washed in S.W. and 4-drop samples were added to 5 ml. *Lytechinus* sperm at the dilutions indicated. Sperm dilution 1 = 16% of undiluted semen, approximately 2×10^8 sperm/ml. in this case.

* Percentage of eggs counted showing the abnormal cleavage characteristic of polyspermy.

pretreatment results in considerable improvement in fertilizability following exposure to dermal secretion. This improvement is evidently limited at the level of the fertilizability of enzyme-treated control eggs.

In the experiments just described the enzyme-treated eggs were washed free of dermal secretion and inseminated in sea water. If the enzyme-treated eggs are

inseminated directly in the dermal secretion they fail to show the marked improvement in fertilizability. In the discussions given below this reservation concerning the loss of dermal secretion sensitivity following trypsin treatment is assumed.

Two possible explanations for the improving action of proteolytic enzyme pretreatment are evident. The enzyme could destroy the activity of the dermal secretion and thereby protect the eggs from this inhibitor, or the enzyme could act directly upon the egg. Even after several washings sufficient enzyme might be carried over or remain adsorbed to the egg to prevent the inhibition by destroying the dermal secretion. This explanation might suffice for the experiments with protease-treated eggs. However, prior to exposure to dermal secretion the trypsin-treated eggs were exposed to an amount of trypsin inhibitor (ovomucoid, four experiments with *Arbacia* eggs; soybean inhibitor, one experiment with *Lytechinus* eggs) sufficient to inhibit all trypsin activity. Furthermore, in one experiment the proteolytic enzyme treatment was applied after exposure of the egg to dermal secretion. This post-treatment with enzyme was as effective as pretreatment in rendering dermal secretion-treated eggs fertilizable. Furthermore, in preliminary experiments the fertilization inhibiting action of dermal secretion was not appreciably affected by long exposure to trypsin. Clearly, then, trypsin does not render eggs insensitive to dermal secretion by inactivating this inhibitor. The alternative, second possibility is that the enzyme destroys or removes some dermal secretion sensitive site or substance of the egg which is essential in normal fertilization. Such action in turn evidently exposes some alternative pathway for fertilization. This alternative pathway is reversibly inhibited by dermal secretion because enzyme-treated eggs fail to fertilize when inseminated in dermal secretion.

Relation of dermal secretion action to the block to polyspermy. The response of enzyme-treated eggs following dermal secretion treatment paralleled the enzyme-treated sea water controls not only quantitatively but also qualitatively. As is well known, eggs treated with proteolytic enzymes undergo polyspermic development at sperm concentrations considerably lower than that required to obtain polyspermy in normal eggs. This susceptibility to polyspermy was not confined to enzyme-treated control eggs. Thus, in the enzyme-pretreated group of experiment 2, Table III, dermal secretion-treated eggs yielded at least as high an incidence of abnormal (polyspermic) cleavage as the enzyme-treated sea water controls. This was true at all sperm dilutions.

These observations suggest that the site of action of dermal secretion may be associated in some way with the block to polyspermy. Accordingly it seemed of interest to test eggs pretreated with another polyspermy inducing agent for sensitivity to the fertilization inhibiting action of dermal secretion. Nicotine has long been known to be a very effective polyspermy inducing agent (see Rothschild, 1953; Hagström and Allen, 1956, for literature) and was therefore selected as the polyspermy inducing agent. Nicotine diluted to 2×10^{-3} in sea water proved to be optimal for inducing polyspermy in *Arbacia* eggs. However, in two experiments such treatment did not lower the sensitivity of *Arbacia* eggs to the fertilization inhibiting action of dermal secretion. When tested quantitatively over a wide range of sperm concentrations, the nicotine-pretreated eggs showed the same degree of fertilization inhibition as control eggs after exposure to dermal secretion. Apparently, then, the sensitivity of eggs to the dermal secretion is not invariably associated with the block to polyspermy.

Relation of the fertilization inhibiting action of dermal secretion to fertilizin. As suggested above, the fertilization inhibiting action of dermal secretion and its reversal by proteolytic enzymes is most readily explained by assuming a combination of the inhibitor with an essential egg substance. Such combination blocks the essential substance and prevents it from entering into reactions involved in the initial stages of fertilization. The substance that immediately suggests itself is fertilizin, and especially so since dermal secretion prevents combination of fertilizin with sperm by inactivating the sperm receptor sites of the fertilizin molecule (Metz, 1959a). If the dermal secretion inhibits fertilization by an essentially irreversible combination of the inhibitor with fertilizin at or near the egg surface, then the fertilization inhibiting action of the dermal secretion should be destroyed by addition of an excess of fertilizin.

TABLE IV

Effect of fertilizin on fertilization inhibiting action of dermal secretion

D.S. dilution	D.S. diluted in fertilizin		D.S. diluted in S.W.		S.W. + fertilizin		S.W. alone	
	Cleavage	Sperm agglutination	Cleavage	Sperm agglutination	Cleavage	Sperm agglutination	Cleavage	Sperm agglutination
1/2	0% (200+)	−	0% (200+)	−	99% (136)	++++	100% (200+)	+
1/10	0% (200+)	+++ ++++	0% (200+)	±				
1/50	13% (159)	++++	6% (140)	+				
1/250	97% (119)	++++	86% (131)	+				

Aliquots of a dermal secretion preparation were diluted serially in sea water and in fertilizin as indicated, two drops of unfertilized *Arbacia* eggs were then added to 0.8-ml. samples of each D.S. dilution, and finally 5 ml. 0.0003% *Arbacia* sperm were added to each sample. % = per cent cleavage; values in parenthesis are the number of eggs counted. To test for fertilizin excess in the mixtures 2-drop samples of the supernatant from each mixture were withdrawn after insemination of the eggs. These were mixed with 2 drops 1–2% sperm and the agglutinating action recorded on a − to ++++ scale in the table. The sperm agglutination titer of the original fertilizin solution was >729.

To test for such inactivation of the fertilization inhibiting action of the dermal secretion by fertilizin, three experiments using *Arbacia* eggs were performed. In these experiments dermal secretion was diluted serially in fertilizin, constant amounts of eggs and sperm were added to the mixtures and the percentage of cleavage determined and compared with appropriate controls. The mixtures were also tested for presence of excess fertilizin by examining for sperm agglutinating action. One experiment, typical of the three, is given in Table IV. As seen in the controls the concentration of sperm used was sufficient to fertilize 99% of the eggs. However, it was necessary to dilute the dermal secretion to 1/250 to approach this value. Furthermore, the dermal secretion inhibited fertilization to the same dilutions (1/50 to 1/250) whether diluted in fertilizin or sea water. Indeed, at dilution 1/50, five times the dilution required to achieve fertilizin excess, only 13% of the eggs fertilized. This parallel loss of fertilization inhibiting action

when diluted in fertilizin and sea water, combined with the demonstration of a fertilizin excess at 1/10 dilution, clearly shows that the fertilization inhibiting action of dermal secretion is not impaired by fertilizin. Evidently, then, fertilizin does not neutralize the fertilization inhibiting action of dermal secretion. Therefore, it seems unlikely that dermal secretion inhibits fertilization by combination with fertilizin.

It should be noted that fertilizin treatment alone did not have a marked effect on fertilization in these experiments. This is in keeping with previous observations on *Arbacia* (Tyler and Metz, 1955). However, fertilizin treatment does have a marked effect in some other species (Tyler, 1941).

Discussion

The experiments described here show that the dermal secretion from *Arbacia* inhibits fertilization by action on the egg, not the sperm. The agent presumably blocks an essential reaction or reactions in the initial stages of fertilization. One such response of the egg that is evidently blocked by the dermal secretion is the formation of the fertilization membrane. In fact, treatment with dermal secretion a few seconds after fertilization arrests membrane formation. Such eggs have membrane elevated over only a part of the egg surface. It will be of interest to determine if other cortical phenomena of fertilization, such as cortical granule discharge (Motomura, 1941; Runnström, 1949; Endo, 1952) and the changes in optical properties observed in dark field illumination, are also arrested by the dermal secretion. These observations should give some indication of whether the agent inhibits the propagation of the cortical responses of the egg or interferes only with the process of membrane elevation (see Metz, 1957, for interrelations of cortical phenomena).

Unfortunately, the relationship of the blebs formed by inseminated oocytes to stages of fertilization of normal eggs is somewhat obscure. Therefore, no detailed interpretation of such inhibition in terms of fertilization is warranted. However, the blebs suggest that some of the initial stages of fertilization have taken place. Accordingly, inhibition of bleb formation by the dermal secretion indicates that this agent can inhibit initial steps in fertilization. It is not unlikely that the dermal secretion inhibits essential steps in the interaction of the sperm and the eggs, as well as arresting manifestations of the propagated responses of the egg.

The fertilization inhibiting action of the dermal secretion presumably results from interaction of the inhibiting agent with some essential substance or substances of the egg. Such interaction evidently results in destruction or blockage of the essential substance. This in turn reduces the number of receptor sites on the egg and the probability of a successful sperm-egg contact. This view is consistent with the observation that inhibitor-treated eggs can sometimes be fertilized if inseminated with high sperm concentrations.

Cases of cleavage delay may also be explained in formal fashion as a reduction of the probability of a successful sperm-egg collision. Thus eggs with a reduced number of receptor sites should have a lower "fertilization rate" (*e.g.* Rothschild, 1956; Hagström and Hagström, 1954) than control eggs because of the increased time required for the less probable event. Another possibility is that cleavage delay is a manifestation of a reversal of inhibition such that washed dermal se-

cretion-treated eggs undergo a slow reversal of inhibition. It should be noted, however, that other factors such as delay in the propagated response of the egg have not been eliminated as explanations for delayed cleavage of dermal secretion-treated eggs.

The fertilization inhibiting interaction between the dermal secretion and the essential egg substance could result in enzymatic destruction of the latter. It appears more likely, however, that the inhibitor acts by relatively undissociable combination with an egg substance. This view is supported by the observation that washing sometimes results in at least partial restoration of fertilizability to dermal secretion-treated eggs. It is also consistent with the observed reversal of inhibition by proteolytic enzymes.

The identity of the egg substance or substances with which the dermal secretion combines to inhibit fertilization remains obscure. This material is evidently not the sperm isoagglutinin, fertilizin. Several lines of evidence support this view. Fertilizin constitutes the jelly layer surrounding the egg. Eggs from which this jelly has been removed by acid sea water treatment are dermal secretion sensitive. Evidently, then, fertilization inhibition is not mediated by action of the dermal secretion on the jelly. However, this experiment does not rule out the possibility of action on an acid-resistant layer of fertilizin bound to the egg surface (*e.g.* Tyler, 1941). Further "dissection" of the egg surface was achieved by treating eggs with proteolytic enzymes. Following such treatment the eggs were relatively insensitive to the fertilization inhibiting action of dermal secretion. Evidently the enzyme digests or otherwise eliminates the primary egg substance with which the dermal secretion combines. However, there is some question if even the relatively drastic action of proteolytic enzymes removes all the fertilizin from the egg surface (Tyler and Metz, 1955) in a reasonable time. As a final test for a role of fertilizin in the inhibition of fertilization by dermal secretion, fertilizin and dermal secretion were mixed and the mixtures were assayed for fertilization inhibiting action. The fertilizin had no effect on the inhibiting action of dermal secretion. This was true even when the fertilizin was present in large excess as measured by sperm agglutinating action.

In view of these results it appears that the dermal secretion does not inhibit fertilization by an action on fertilizin. This is of particular importance because previous studies (Metz, 1959a) have shown that dermal secretion of *Arbacia* destroys the sperm combining sites of the fertilizin molecule.

Apart from consideration of the nature of the egg substance involved in the inhibition, the reversal of inhibition by either "pre- or post-" treatment of eggs with proteolytic enzymes has interesting implications for other aspects of fertilization. According to the interpretation given above dermal secretion inhibits fertilization by combination with a substance that is essential for fertilization of the normal egg. Pretreatment of the egg with proteolytic enzymes removes this essential egg substance and renders the egg relatively insensitive to the inhibitor. Evidently coincident with the removal of the essential egg substance by enzyme, an alternative pathway(s) to fertilization is exposed. Whether this pathway resides in the sperm-egg attachment mechanism, the activation initiating mechanism or the propagative and cortical response mechanism remains to be established. However, it should be noted that the "alternative pathway" exposed by proteolytic

enzyme treatment is associated with a loss of the block to polyspermy and a reduction in fertilization specificity (Bohus Jensen, 1953; Hultin, 1948a and 1948b; Tyler and Metz, 1955) and is itself reversibly inhibited by dermal secretion. This last conclusion follows from the fact that enzyme-treated eggs must be washed free of dermal secretion before they will fertilize. Whatever the site of action of the proteolytic enzymes the action does not result in drastic visible effects on the unfertilized egg, for sections of trypsin-treated and control eggs are indistinguishable when seen with electron optics (Parpart, personal communication).

This investigation suggests the use of fertilization inhibiting agents as markers or labels for particular sites or substances involved in fertilization. Extension of the investigation to include detailed examination of other fertilization inhibitors should prove interesting for these may act at other sites. Indeed, the studies of Branham and Metz (1959) indicate that the inhibition of fertilizin agglutination of sperm by extracts from *Fucus* and by dermal secretion results from two quite different mechanisms. It will not be surprising if these two agents are found to inhibit fertilization by action at different sites in the complex of reactions that result in fertilization.

Summary

1. Sperm washed from *Arbacia* dermal secretion fertilize eggs as readily as sperm washed from sea water.

2. Eggs washed from *Arbacia* dermal secretion do not fertilize as readily as controls. At best such eggs require high sperm concentrations to achieve fertilization. In some experiments the treated eggs show a marked cleavage delay. It is concluded that the dermal secretion inhibits fertilization by action on the egg, not on the sperm.

3. Addition of dermal secretion to eggs several minutes after insemination has no effect on the development of the eggs. Similar treatment a few seconds after insemination results in arrest of fertilization membrane elevation and sperm penetration. The dermal secretion also inhibits the formation of blebs in inseminated oocytes. It is concluded that dermal secretion inhibits by blocking an initial stage(s) in fertilization.

4. Jellyless (acid-treated) eggs fail to fertilize after treatment with dermal secretion. They have undiminished sensitivity to the inhibiting action of the dermal secretion.

5. Proteolytic enzyme-treated eggs are relatively insensitive to the inhibitor. They fertilize as readily as controls after treatment with dermal secretion, provided they are washed free of this inhibitor. They fail to fertilize if inseminated in dermal secretion.

6. The dermal secretion has undiminished fertilization inhibiting action in the presence of an excess of fertilizin.

7. The experiments suggest that the dermal secretion inhibits fertilization by combining with some egg substance that is essential for fertilization of the normal egg. The egg substance is apparently not the sperm isoagglutinin, fertilizin. Treatment of the egg with proteolytic enzymes eliminates the essential egg substance and simultaneously exposes an alternative pathway to fertilization. The latter is reversibly inhibited by dermal secretion.

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